A COMPACT DEVICE FOR DIGITAL DROPLET PCR

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ABSTRACT

In this work, the fabrication and testing of a miniaturized device to perform all steps necessary for digital droplet polymerase chain reaction (ddPCR) within a single chip is presented. The microfluidic chip, made in silicon, contains a T-junction for droplet generation along with a microreactor for temperature cycling. The temperature is controlled by means of an external, miniaturized thermoelectric element. The number of fluorescent droplets after PCR amplification is determined using a fluorescent microscope; furthermore, individual droplet fluorescence is recorded at each cycle, thus enabling real time PCR analysis. The microreactor accommodates about 2500-3000 droplets of uniform volume varying in the 55-65 pL range, allowing the direct and accurate detection of human genomic DNA in concentrations from 0.06 to 40 ng/µL.

KEYWORDS: DNA quantification, digital droplet PCR

INTRODUCTION

Quantitative PCR (qPCR) is the golden standard for nucleic acid quantification. Although qPCR is a robust and well established technique, it suffers from some drawbacks. The most important is the need to perform a series of experiments to provide accurate calibration curves. This is a lengthy process and to be accurate requires the availability of samples identical to the one to be analyzed with known concentration of nucleic acid, which is not always possible.

ddPCR is an emerging alternative method enabling a direct quantification of nucleic acids. It is based on partitioning the sample into small droplets, each initially containing at most a few units of the template. After PCR, the ratio of positive (detected by fluorescence) to total partitions allows, through the use of Poisson statistics, an accurate determination of the average template number per partition before amplification. Based on the known partition volume the template concentration can be calculated. Available commercial solutions for ddPCR propose a lengthy workflow using different tools for sample partitioning, temperature cycling, and fluorescence reading [1].

In this work, we present a miniaturized ddPCR chip, based on a Si microfluidic platform developed at imec [2]. The chip combines uniform droplet generation and fast temperature cycling, while optical readout is accomplished using a fluorescence microscope.

CHIP FABRICATION AND EXPERIMENTAL SETUP

The ddPCR chips were fabricated in silicon using deep reactive ion etching. Sealing of the microfluidic structures was performed by anodic bonding of Pyrex to ensure optical access. After bonding, a second silicon etch was performed on the backside of the wafer to define holes for fluidics access and trenches for thermal insulation of the microreactor. Details of the fabrication process have been published before [2]. Figure 1 shows a photograph and the layout of the fabricated ddPCR chips. Channels forming the droplet generators are 30 or 50 µm wide, while the S-shaped PCR reactor occupies a 3mm x 3mm area. Devices with a different etch depth of 30 µm or 60 µm were fabricated, corresponding to a microreactor volume of 0.2 µL or 0.4 µL. Data reported hereafter refers to the 30 µm deep devices.

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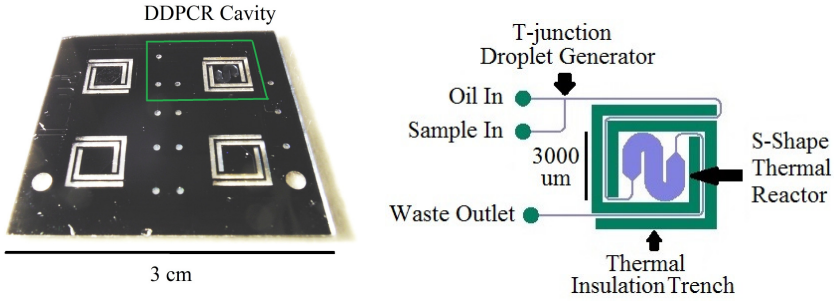
Droplets from the PCR sample were generated in fluorinated oil mixed with appropriate surfactants (BioRad 186-3005) using a T-junction channel geometry. The flow rate of the sample and the oil streams were adjusted respectively to 0.4 µL/min and 0.8 µL/min.



(a) (b)

Figure 1. (a) Photograph of a chip containing 4 identical devices. b) Layout of a single device composed of a droplet generator and a thermally insulated cavity. The violet structures are etched from the front side, to a depth of 30 µm or 60 µm. The green structures are etched from the backside to the full wafer thickness.

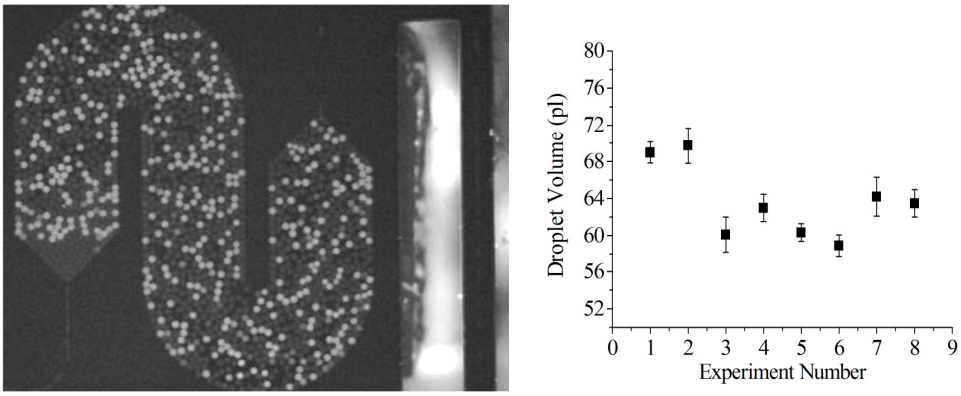
Optical fluorescence read-out was performed using an external light source and a CCD camera coupled to an inverted microscope. The fluorescent image of the cavity was analyzed using ImageJ, an open source image processing software package used to count the number of droplets and measure their size and their fluorescence intensity.

For the ddPCR results presented here, a commercial PCR mastermix (2x PCR Super Mix for probes, 186-3010, BioRad) was used together with a commercial primer and probe set (20x PrimePCR™ ddPCR™ EGFR copy number assay, 100-31240, BioRad) and a variable concentration (from 0.06 to ng/µL to 40 ng/µL) of human genomic DNA as template.

A two-step PCR was performed according to the following scheme: enzyme activation for 5 minutes at 95 ºC, 50 cycles of 95 ºC for 15 s with a ramp time of 6s to 60 ºC, held for 45 s, with ramp time of 12 s back to 95 ºC. Total cycle time was 78 s and total experiment time 70 min.

RESULTS

Fluorescence data were collected from the about 3000 droplets contained in the current version of the PCR microreactor. Figure 2a shows an image of the ddPCR cavity after 50 temperature cycles illustrating the good contrast between the positive and negative droplets.



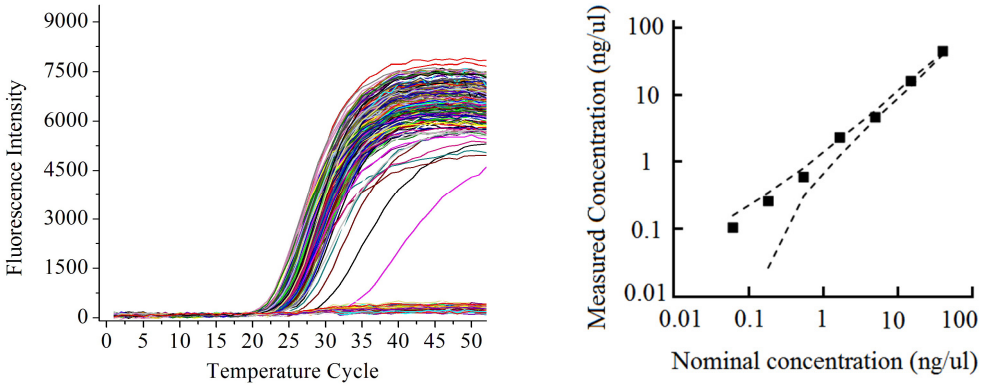
(a) (b)

Figure 2. (a) Photograph of the reactor at the amplification endpoint. (b) Average droplet volume in 8 different experiments.

Droplet size analysis was performed with ImageJ to determine the average droplet volume in a series of eight experiments repeated with the same droplet generation conditions. Results are shown in Figure 2b, the average volume is about 63pL per droplet and the volume variation, calculated from standard deviation is less than ±5%. The droplet generation rate, calculated as the ratio of the volumetric flowrate to the droplet volume, is about 6400 droplets per minute.

The intensity analysis at each cycle provides real-time PCR curves for each droplet. In Figure 3a an example of these data is given for a sample containing 15 ng/µL human genomic DNA: traces corresponding to positive and negative droplets are clearly separated.

To demonstrate the functionality of the integrated device, human genomic DNA with concentrations ranging from 0.06 to 40 ng/µL was amplified. The concentration values obtained from the experiment are plotted against the nominal ones in Figure 3b. The good correspondence over three orders of magnitude illustrates the power of ddPCR for accurate DNA quantification. In the experiment with the lowest concentration of DNA only a few droplets were fluorescent, which sets the lower limit on the measured concentration. The largest measured concentration corresponds to about 0.6 template per droplet and, in theory, can be further increased. A template concentration value 5 times larger would still give a measurable number of non-fluorescent droplets (about 150 out of a total of 3000). We can then conclude that the limits on the dynamic range of the fabricated devices is close to four orders of magnitude.



(a) (b)

Figure 3. (a) Droplet fluorescence intensity vs. cycle number for all droplets. Human genomic DNA concentration was 15 ng/µL (b) Measured concentration vs. expected one. The dotted lines are the 95% confidence interval.

CONCLUSION

In conclusion, an on chip ddPCR microsystem was demonstrated that paves a route toward integrated, fast and accurate quantification of nucleic acids. In future implementations the dynamic range can be extended by increasing the volume of the system; a 100 fold increase can be obtaining by increasing both the depth and footprint area by a factor of 10, which still results in a compact device (10 mm x 10mm).

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